Reorganization of Keratin Intermediate Filaments by the Drug-Induced Disruption of Microfilaments in Cultured Human Keratinocytes

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It has been shown to date that a combined treatment with microtubule and microfilament inhibitors alters the cytoskeletal organization of keratin intermediate filaments in cultured HeLa, fetal mouse epidermal, and epithelial PtK_2 cells, although neither of these inhibitors alone is able to do so. In the present study, we found that disruption of microfilaments with cytochalasin B induced a remarkable reorganization of keratin filaments in cultured human keratinocytes, while disruption of microtubules with colchicine did not affect keratin filaments. Keratin filament organization in the presence of cytochalasin B demonstrated a network of connecting star-like knots or foci. These foci coincided with actin aggregates that were formed by depolymerization of actin filaments as studied by double immunofluorescence using antiactin and antikeratin antibodies. Under these conditions, no change in microtubule arrangement was observed. Our observations suggest that the stability and architecture of keratin filament organization may be supported with the microfilament rather than the microtubule cytoskeleton in cultured human keratinocytes. *J Invest Dermatol 87:565–569, 1986*

Keratin filaments are the intermediate-sized filaments of epithelial cells, and form one of the 3 classes of cytoskeletons, along with microfilaments and microtubules, in these cells (reviewed in [1,2]). Recently, the biochemical and immunologic characteristics of keratin filaments have been reviewed [3,4]. However, the function of keratin filaments as the intermediate filaments in epithelial cells has not yet been clearly defined, although they may function to support mechanically the various structures of the cytoplasmic space [1].

In order to characterize the dynamic functions of keratin filaments, studies of the rearrangement of these filaments during mitosis have been performed [5–15]. These studies have indicated that keratin intermediate filaments are systematically and dynamically rearranged during mitosis and, although not all epithelial cell types undergo this phenomenon, they may play some important roles in mitosis.

Besides these studies, some experiments have been conducted to establish methods to disrupt the structure and functions of keratin intermediate filaments. There are no biochemical agents that are known to specifically destabilize the structure and/or interfere with the functions of keratin and other intermediate filaments in a manner analogous to that of the cytostatic drugs such as colchicine and cytochalasin B, which act on microtubules and microfilaments, respectively. Recently, a possibility that acrylamide disrupts reversibly the keratin network in PtK_1 cells has been reported [16]. Microinjection of anti-α-keratin antibodies into epithelial cells has also been used for the same purpose, showing that the selective destabilization of the filamentous networks of keratin occurs without changing cell shape in PtK_1, PtK_2, and SV-40 transformed human keratinocytes [17]. A combined treatment with microtubule and microfilament inhibitors has also been used successfully to induce the reorganization of the cytoskeletal array of keratin filaments in cultured HeLa and fetal mouse epithelial cells [18,19], although disruption of microfilaments with cytochalasins [18,19] or microtubules with colchicine derivatives [19,20] did not significantly alter the inherent organization of keratin filaments. However, it is of great interest to note that both cytochalasin B alone and demecolcine alone altered keratin filament organization in epithelial monkey kidney TC7 cells [21].

In the present study, it is shown that the disruption of microfilaments with cytochalasin B alone induces a remarkable reorganization of keratin filament networks in cultured human keratinocytes, while the disruption of microtubules with colchicine does not.

MATERIALS AND METHODS

Antibodies Polyclonal antibodies against keratin filament proteins of human epidermis, which were obtained by immunizing a rabbit with a 50 kD subunit of human epidermal keratin [13], were used. Monoclonal antibodies (mouse IgG) against α-tubulin or actin were purchased from Amersham International (England). As secondary antibodies, fluorescein isothiocyanate (FITC)- or rhodamine-lissamide sulfonyl-chloride-labeled goat IgG anti-mouse IgG, and FITC-labeled goat IgG antirabbit IgG, which were purchased from Cappel Laboratories (Westchester, Pennsylvania), were used in the present study.

Cell Culture After removing most of the subcutaneous tissue with surgical scissors, skin biopsies from human foreskin or other sites were cut into small pieces (2 × 5 mm) and soaked in a solution of 1000 U/ml dispase (Godo Shusei Co., Matsudo, Chiba,

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Abbreviations:
DMSO: dimethylsulfoxide
FITC: fluorescein isothiocyanate
PBS: phosphate-buffered saline
PMSF: phenylmethanesulfonyl fluoride
TPCK: L-1-tosylamide-2-phenylethyl chloromethyl ketone

Japan) in phosphate-buffered saline (PBS) for 15 min at 37°C. After this incubation, the epidermis could be easily peeled off the dermis with forceps. The epidermis was then incubated in 0.25% trypsin and 0.05% EDTA for 10–15 min at 37°C to obtain dispersed keratinocytes. Approximately 10^5 keratinocytes were inoculated on glass coverslips in a 35-mm plastic dish. The cultures were fed with Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum, 0.4 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 84 ng/ml cholera toxin, 100 μg/ml streptomycin, and 100 U/ml penicillin [22]. The medium was changed at 2-day intervals. Cultures were grown in a humidified atmosphere under 5% CO_2 at 37°C. Cultured cells were subjected to experiments 4–6 days after plating.

Treatments with Colchicine and Cytochalasin B Cytochalasin B and colchicine (Sigma Chemical Co., St. Louis, Missouri) were dissolved in DMSO and in PBS, respectively, and added in the medium to obtain the final concentration of 20 μM for each. The cells were incubated in these media for 30, 60, and 120 min and then fixed with cold methanol (−20°C). For control experiments, the cells were incubated in the medium containing DMSO without cytochalasin B and colchicine for the same periods. Cell viability during experimental periods was over 90% as judged by the trypan blue exclusion test.

Immunofluorescence Microscopy The cells grown on the coverslips were rinsed with PBS at room temperature, and dipped for 7 min in methanol at −20°C. The fixed cells, on the coverslips, were soaked in a solution of 0.5% Triton X-100, 2 mM phenylmethanesulfonyl fluoride (PMSF) and 2 mM L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) for 10 min at room temperature.

The cells were incubated with the first antibody for 45 min at room temperature, followed by several washes in PBS. In some specimens, double antibody labeling was carried out as follows: first the cells were incubated with rabbit antibody against keratin for 45 min followed by extensive washing, then with the monoclonal antibody against α-tubulin or actin for 45 min. After incubation with the first antibodies and washing, the cells were incubated with the specific second antibodies, rinsed with PBS, air-dried, and mounted in glycerol. Controls for specificity of the antibodies were routinely carried out. Photomicrographs were taken with Nikon fluorescence microscope VFD-R (Nikon, Tokyo, Japan).

RESULTS

The cytoskeletal arrangement of keratin filaments in cultured human keratinocytes, which was visualized by immunofluorescence microscopy using antikeratin antibodies, showed a dense meshwork around the nucleus and a radiating pattern of filament bundles to the cell periphery (Figs 1*a*, 2*a*). Microtubules also showed an array similar to that of keratin, but they appeared to form much thicker filament bundles (Fig 1*b*). The fluorescence pattern of actin filaments by using antiactin antibody is shown Fig 2*b*, which showed diffuse distribution of actin filaments throughout

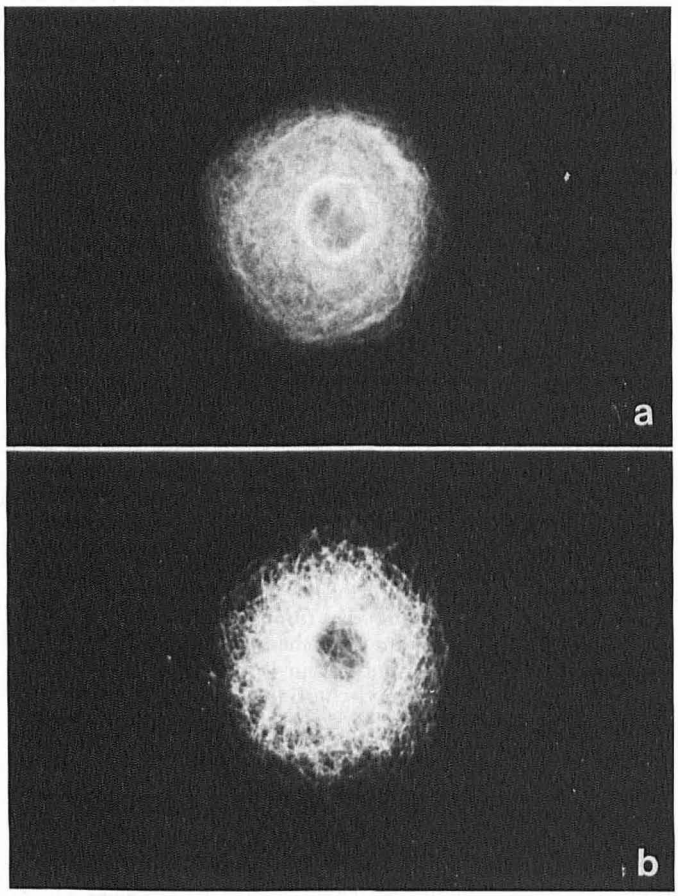

Figure 1. Double-labeled immunofluorescence localization of keratin filaments (*a*) and microtubules (*b*) in cultured human keratinocytes is demonstrated by using antikeratin and antitubulin antibodies. These are untreated control cells. Both cytoskeletons show a radial pattern from juxtanuclear filament aggregations.

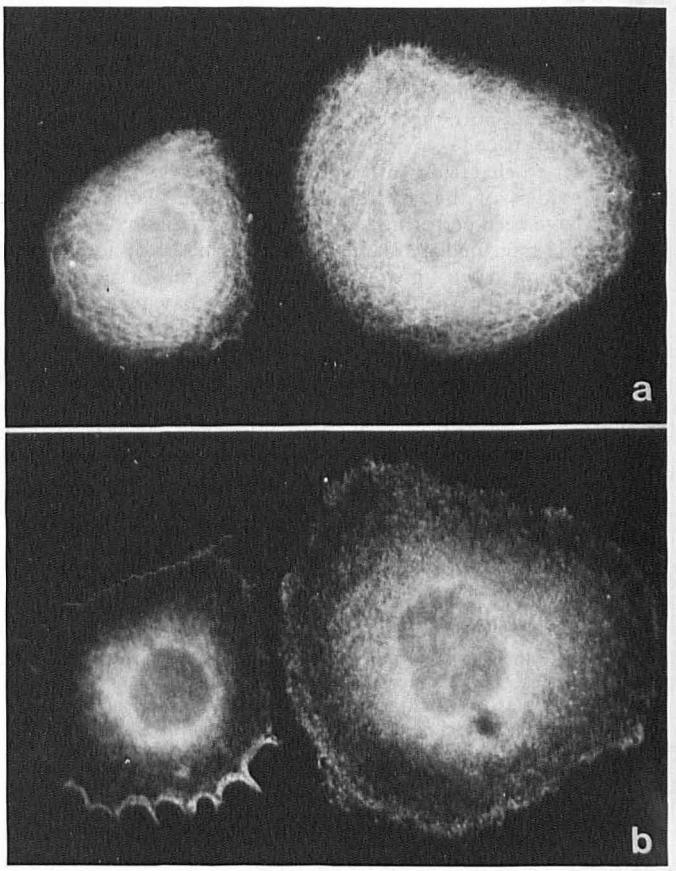

Figure 2. Double-labeled immunofluroescence distribution of keratin filaments (*a*) and microfilaments (*b*) in cultured human keratinocytes is shown by using antikeratin and antiactin antibodies. These are untreated control cells. Keratin shows a fibrillar network (*a*) but actin shows a denser staining pattern in juxtanuclear and cell periphery regions (*b*). Actin filaments are not clear by immunofluorescence.

the cell, although filament structures were not resolved by this method.

When the cells were treated with cytochalasin B, the diffuse distribution of actin filaments was disrupted and condensed into small aggregates in the cells as shown in Fig 3*b*, and keratin filaments were also rearranged into a unique pattern, which was a "lattice" structure consisting of many foci of small asteroid aggregations of filament bundles (Fig 3*a*). These foci of keratin-filament assemblies appeared to coincide with the small aggregates of actin as indicated by arrows in Fig 3*a* and *b*. Observations at higher magnification revealed that the centers of some of these knots were not stained with antikeratin antibody, but they were solidly stained with antiactin antibody (Fig 3*a* and *b* insets). This rearrangement of keratin filaments did not induce the detachment of desmosomal cell-to-cell contact. Keratin filaments formed aster-like foci also at the cell contact region (*large arrow*, Fig 3*a*). Although the keratin filaments were rearranged into the lattice structure, no significant rearrangement of microtubules was detected by the double-immunofluorescence method (Fig 4*a* and *b*). These alterations of the keratin filament and actin organization were observed in 60–90% of the cells in culture dishes in different experiments, when almost all cells on the coverslips were examined.

The disruption of microtubules with colchicine did not induce any significant reorganization of keratin intermediate filaments (not shown). Combined treatment with cytochalasin B and colchicine produced a lattice arrangement of keratin filaments almost identical to that produced by cytochalasin B treatment alone (not shown).

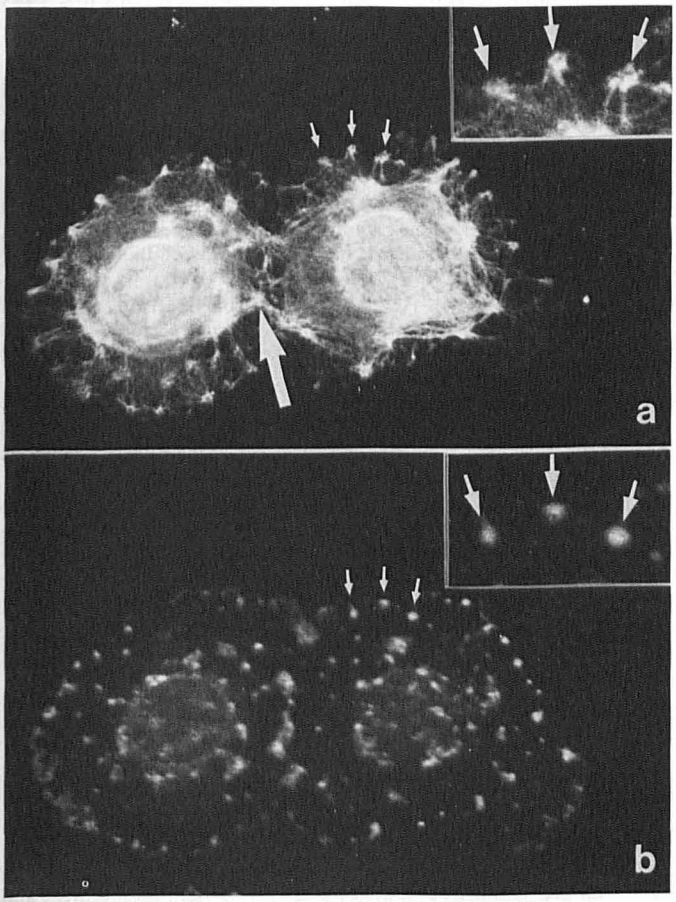

Figure 3. Effects of a 1-h treatment with cytochalasin B (20 μM) on the keratin (*a*) and actin (*b*) filament cytoskeletons in cultured human keratinocytes is shown by double-labeled immunofluorescence (antikeratin, and antiactin antibodies). The keratin filament arrays are re-formed into a star-like, knotted keratin network (*a*), while actin filaments are resolved into small punctate aggregates (*b*). The keratin knots appeared to coincide with actin (*arrows*). The center of keratin foci appears to be unstained (*arrows, inset*).

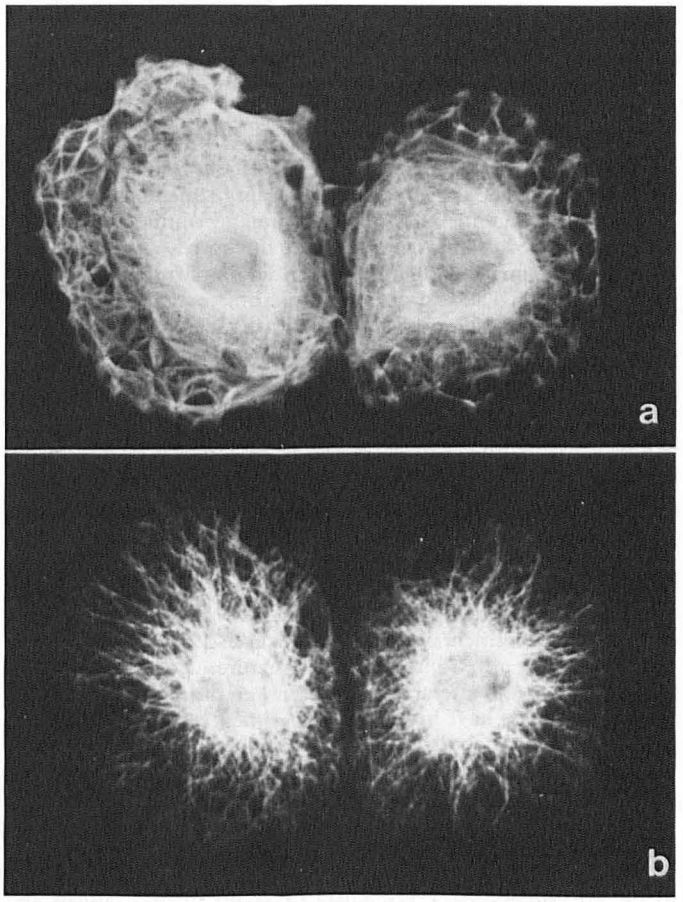

Figure 4. Effects of a 1-h treatment with cytochalasin B (20 μM) on keratin filament (*a*) and microtubule cytoskeleton (*b*) is shown by double-labeled immunofluorescence using antikeratin and antitubulin antibodies. There are no major effects on the microtubular cytoskeleton by reorganization of keratin filaments exerted by cytochalasin B-induced microfilament disruption. A 1-h treatment with cytochalasin B changed the keratin filament arrangement into a star-like, lattice structure (*a*), but did not change microtubule organization (*b*).

DISCUSSION

The present study has shown that the disruption of microfilaments by cytochalasin B induces a reorganization of keratin filament networks in cultured human keratinocytes in the absence of major reorganization of microtubules (Fig 4*a* and *b*). Similar effects of cytochalasin B on keratin filament networks have been observed in monkey kidney TC7 cells [21]. However, the TC7 cells respond to demecolcine-induced disassembly of microtubules so as to reorganize keratin filament arrays, although human keratinocytes do not respond in this manner to microtubule disassembly (induced by colchicine in the present study). On the other hand, it has been shown that the combined disruption of microtubule and microfilament cytoskeletons alters the keratin cytoskeletal arrangement in mouse carcinoma cells, mouse epidermal cells, and HeLa-S_3 cells [18,19]. These papers have described that addition of any one drug alone or of cytochalasin D and β-lumicolchicine simultaneously does not elicit significant organizational change in the keratin cytoskeleton and suggested that microfilaments and microtubules have a combined role in maintaining the arrangement of keratin in HeLa and mouse epidermal cells.

In epithelial PtK_2 cells also, combined treatment with cytochalasin D and colcemid induced a star-like, lattice-like redistribution of keratin filaments, but neither colcemid nor cytochalasin D alone was able to cause a major reorganization of keratin filaments [20].

Since the microfilament disruption induced the keratin filaments to form a lattice structure connected with star-like knots, which are also stained with antiactin antibody (Fig 3*a* and *b*), it is probable that the organization of keratin filaments is supported with actin arrangements in cultured human keratinocytes. However, when the keratin filaments were disrupted with antikeratin antibody, the microfilamentous array did not change in PtK_1 and PtK_2 cells [17]. The disruption of microfilaments alone did not change the keratin filament organization in HeLa cells, fetal mouse epidermal cells, PtK_1 and PtK_2 cells [18–20,23]. Therefore, it appears that the keratin cytoskeleton has different relationships with microfilament systems among different cell types.

The knots of star-like arrangements of keratin filaments showed what appeared to be central unstained spots (Fig 3*a* and *b*), while these knots are stained completely with antiactin antibody. This suggests that keratin filaments may aggregate or focus onto the small mass of actin or may be trapped at the sites common to actin aggregation. In this respect, it may be worthwhile to note that intermediate filaments (vimentin or keratin) in PtK_1 cells appeared to be interwoven into masses of unordered microfilaments after treatment with cytochalasin B [24], and that they were not reorganized into a lattice structure.

Concerning the interaction between keratin filaments and microtubules, there have been some experiments which suggest that intermediate filaments may depend on microtubules for their distribution in the cytoplasm and may act in coordination with microtubules in the positioning of nuclei. This situation has been observed in SV5-infected BHK21-F syncytia [25], although an agent, vanadate, has been found that separates intermediate filaments and microtubules topologically and functionally [24]. Also, the collapse of intermediate filaments during mitosis and after drug-induced microtubule depolymerization suggests the dependence of intermediate filament organization on microtubules in chick gizzard cells [26]. Recently, intermediate filaments (vimentin) in gerbil fibroma cells have been shown to be collapsed in living cells microinjected with monoclonal and polyclonal antibodies against tubulin [27]. It has been suggested from these data that the antibodies disrupted postulated intermediate filament-microtubule interactions [27].

Our results, however, showed that the organization of keratin filaments appeared to be independent of the microtubule cytoskeleton, since the reorganization of keratin filaments by disruption of microfilaments did not significantly affect microtubule organization (Fig 4*a* and *b*), and the depolymerization of microtubules did not change the keratin filament networks in cultured human keratinocytes. The independence of the organization and reorganization of keratin filaments from microtubules also has been suggested by double-labeled immunofluorescence microscope studies on alterations of these cytoskeletons during mitosis [5–13] in human keratinocytes [13] and other epithelial cells [5–13]. The reason for the difference in susceptibility of keratin filaments in human keratinocytes to disruption by depolymerization of microfilaments and microtubules are unknown.

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